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Genetic and molecular characterization of *Candystripe1* transposition events in sorghum

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Abstract

In sorghum, the *Candystripe1* (*Cs1*) transposable element causes a variegated pericarp phenotype due to its excision activity from the *y1 (yellow seed1)* locus. The Y1 is a transcription regulator which is required for the biosynthesis of red 3-deoxyflavonoid pigments. Somatic variability in the transposition behavior of *Cs1* was observed via biochemical analysis of 3-deoxyflavonoids in the leaf tissues of the *Y1-cs* alleles. Using somatic excisions of *Cs1* as a tool, we establish that the *Cs1* is active in young seedlings and the *y1* locus is also functional in these tissues. Several somatic and germinal excision events were characterized and sequence analysis of independent events predominantly showed 2-bp footprints. Further, with the goal of understanding the properties of *Cs1* that would facilitate the development of a transposon tagging system in sorghum, germinal excisions of *Cs1* from *y1* were used as a marker. Transposition of *Cs1* was followed by characterization of putative insertion events. Genetic linkage between mutant phenotypes and the co-segregating restriction fragments of *Cs1* provided additional evidence that *Cs1* is an active transposable element in sorghum.

Introduction

Sorghum, a C4 grass that diverged from maize about 20 million years ago (Gaut & Doebley, 1997), is the fourth most important cereal crop grown worldwide. This grain and forage crop is important because of its unusual tolerance to hot and dry environments. Thus, sorghum has been identified as a key plant species for the comparative analysis of grass genomes, and as a source of beneficial genes for agriculture (Mullet, Klein & Klein, 2002). Sorghum's relatively small genome of 750 million base pairs (Arumuganathan & Earle, 1991), agronomic trait diversity in the germplasm (Djè et al., 2000; Kong, Dong & Hart, 2000), incremental divergence from maize and rice (Doebley et al., 1990), low amount of repetitive DNA and co-linearity with other cereal genomes (Bennetzen et al., 1998; Gale & Devos, 1998) makes it ideally suited for discovery and analysis of grass genes through comparative genomics.

The application of plant transposons to gene tagging was first demonstrated by the cloning of the *bronze* gene in maize using the *Ac* (*Activator*) element (Fedoroff, Furtek & Nelson, 1984). Since then, a growing number of plant genes have been cloned or identified using transposable elements (reviewed in Nevers, Shepherd & Saedler, 1986; Walbot, 1992; Bennetzen, 1996; Gierl, 1996;

Kunze, 1996; Ramachandran & Sundaresan, 2001). Thus, trait characterization at the genetic and physiological levels can be enhanced by the availability of transposon tagged mutations and transposon mutagenesis can be one of the successful genetic tools for isolating genes in sorghum. However, until recently, no active transposable element was known in sorghum. Transposition of modified *Ac*/*Ds* and *En*/*Spm* elements in species such as *Arabidopsis*, tomato and rice have expanded the use of tagging systems in readily transformed species (Jones et al., 1989; Wisman et al., 1998; Tissier et al., 1999; D'Erfurth et al., 2003). Such approaches of introducing *Ac*/*Ds* in sorghum are under way, but are being hampered by several factors including poor insertion rates of *Ds* (Ian Godwin, University of Queensland, personal communication), as well as gene silencing and low efficiency of sorghum transformation (Carvalho, 1999). Thus, for plant species like sorghum, which are also not amenable to routine plant transformation methods, an endogenous active transposable element may provide an efficient means for gene tagging.

In sorghum, the functional *y1* gene encodes a Myb type of transcription factor that controls the biosynthesis of 3-deoxyflavonoids pigmentation (phlobaphenes) pigments in the seed pericarp and in other parts of the plant including mature leaves (Figure 1; Zanta et al., 1994). During our previous study of phlobaphenes biosynthesis regulation in sorghum, we discovered a transposable element. The *Cs1* element was cloned from the 'candystripe' sorghum (Chopra et al., 1999), a sorghum land race which has variegated pericarp phenotype that is genetically linked to the *Y1-cs* (*Y1-candystripe*). Variegations in the pericarp are the result of somatic excisions of *Cs1* from *Y1-cs* in some cells whose progeny produces red stripes against a colorless background. Germinal excision of the *Cs1* gives rise to fully red headed (sorghum inflorescence) plants. We now are further interested in understanding the excision and re-insertional properties of this DNA element that may be used in

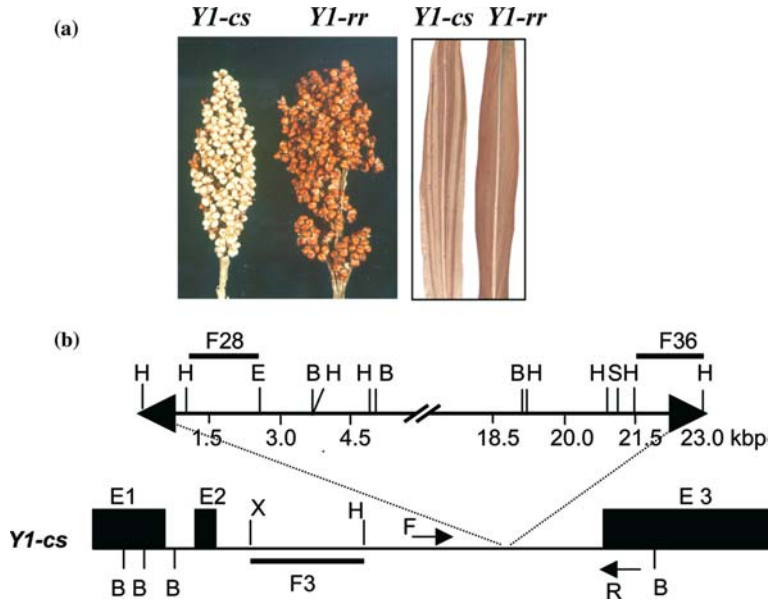

Figure 1. Characterization of *yellow seed1* as a phenotypic and molecular marker for excision of *Candystripe1* transposon in sorghum. (a) Phenotypes of sorghum inflorescence (left) and leaves (right) showing *Y1-cs* and *Y1-rr* alleles. (b) A line diagram showing partial restriction map of the sorghum *Y1-cs* allele (not drawn to scale). Black boxes represent exons that are joined by lines as introns of the *y1* gene. The *Cs1* element present in the second intron of the *y1* sequence is shown as a triangle. The outwardly pointed arrowheads on the termini of the *Cs1* sequence represent 20-bp terminal inverted repeat sequences. Positions of the 5′- and 3′- end DNA fragments of *Cs1* used as probes are shown as F28 and F36. Fragment F3 represents the DNA fragment of *y1* intron II sequence used as a probe to detect polymorphisms. Restriction enzyme sites shown are: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sca*I; and X, *Xho*I. Positions of forward (F) and reverse (R) primers used in the footprint analysis are shown as arrows on the *y1* sequence.

developing a future transposon tagging system in sorghum. In this study, we have used the Y1-regulated pigmentation as a marker in seedlings to genetically link the restriction fragment length polymorphism associated with the somatic excisions of the *Cs1* from *Y1-cs*. We have further isolated and analyzed somatic and germinal excision footprints to compare the behavior of the *Cs1* to that of other CACTA elements. Results are presented on the identification of *Cs1* re-insertional genomic regions, and phenotypic and genetic characterization of putative sorghum mutants.

Materials and methods

Genetic stocks

The original candystripe line (CS8110419) was collected from Gedaref, Sudan by O. Webster as described previously by Chopra et al. (1999). The CS8110419 stock carrying a mutable *y1* allele (designated as *Y1-cs*, candystripe) was crossed with different sorghum inbred lines to improve agronomic traits, and to develop a seed color that would facilitate identification of the red or variegated pericarp phenotypes. For genetic tests, sorghum lines carrying a non-functional *y1* allele with white pericarp and white glume phenotype designated as *y1-ww*, or a functional *y1* allele with red pericarp and red glume phenotype designated as *Y1-rr* were used. Two independently isolated alleles of *Y1-cs* designated as *Y1-cs-3 and Y1-cs-4* were also used in this study to monitor somatic excisions of the *CS-1* element from the *Y1* gene.

Population development, mutant screen, and genetic analysis

Genetic cross CS8110419 × Tx2737 was used for developing recombinant populations for screening of new mutations. To begin with, all F_1 plants were self pollinated and advanced to the F_2 generation. Germinal revertants of the *Cs1* were identified as plants with fully red inflorescence (red pericarp and red glumes). Red revertant as well as any candystripe sibling plants were saved and selfed in each generation and this process continued till F_7. At the same time, new mutations observed were also saved and characterized in further generations to examine their heritability. To test the mobility of the *Cs1* transposon in the early segregating generations, a sub-set of red revertant plants were used for excision and re-insertion in the genome. All of the mutants were identified by visual selection from independent rows, and several mutants showed similar phenotypes. When a mutant phenotype was identified, the row was self-pollinated, and seeds from the mutant and the normal plants were grown in the following season to study the inheritance of the trait. A total of 340 plants belonging to 10 families were screened by DNA gel blot analysis. In the later generations, seeds from each selected red panicle were grown in a progeny row and screened for segregating mutations. Based on the segregation test of a mutation (Table 2), plants were identified for the mutant and wild type phenotypes and were further used for the co-segregation analysis.

Genomic DNA isolation and Southern hybridization

For determination of allelic state of *y1*, young leaves of 10-day-old individual seedlings of the *Y1-cs* allele were ground in liquid Nitrogen and the powder was divided into three equal portions. One part was used for isolation of genomic DNA (Saghai-Maroof et al., 1984), while the other two portions were kept at −80°C for isolation of RNA and extraction of flavan-4-ols (see below). For footprint analysis, genomic DNA from seedling leaves or immature pericarps (18 days after anthesis) was isolated. Restriction digestions were performed using enzymes, reagents and reaction conditions from Promega (Madison, WI). All hybridizations were performed using α-P^{32}-dCTP labeled probes. DNA fragment F3 of the *y1* gene (see Figure 1b for position of probes) was used to perform Southern hybridizations to detect somatic and germinal excisions of the *Cs1* from *Y1-cs*. For RFLP analysis of new mutations, a 1.08-kbp *Hin*dIII-*Eco*RI fragment from the 5′ end (F28), a 1.8 kbp *Hin*dIII fragment from the 3′ end (F36) and additional DNA fragments (data not shown) spanning the whole length of the *Cs1* element were used as probes. DNA gel blot hybridizations were performed using a hybridization mixture containing dextran sulfate (10%), NaCl (1 M), SDS (1%), Tris–HCl (10 mM) and 0.25 mg/ml salmon sperm DNA. Blots were pre-hybridized at 65°C for 6 h in the hybridization buffer without a labeled probe followed by hybridizations for about 20 h at 65°C.

Filters were washed in 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.5% SDS at 50°C for 15 min, and twice at 65°C for 15–30 min. Filters were exposed to X-OMAT film (Kodak) for 1–4 days before developing. Filters were stripped by washing for 15 min in boiling solution of 0.1% SDS before re-hybridization.

RNA isolation and Northern blot analysis

One portion of the tissue sample (see DNA isolation method above) was extracted with TriReagent (Molecular Research Center Inc., Cincinnati, OH). RNA was separated on a denaturing gel containing 5% formaldehyde (v/v), 1.2% agarose (w/v) and 1 × MOPS buffer (0.4 M MOPS, 0.1 M anhydrous sodium acetate and 0.01 M Sodium EDTA) followed by transfer onto a nylon membrane (Osmonics Inc., Minnetonka, MN). A radioactively labeled probe of the y1 cDNA fragment corresponding to exon 3 (Sangar, 2003) was used to detect y1 specific transcripts. RNA gel blot hybridizations were performed for 24 h at 43°C in a hybridization mixture containing 50% formamide, 0.25 M sodium phosphate at pH 7.2, 0.25 M sodium chloride, 1 mM EDTA, 7% SDS, and 0.05 mg/ml sheared salmon sperm DNA (Chopra, Athma & Peterson, 1996). Filters were processed and exposed to X-ray film as explained in the DNA gel blot hybridization section.

Analysis of flavan-4-ols accumulating in sorghum seedlings

One hundred milligram of seedling tissue was incubated in 500 µl of cold methanol for 24 h at 4°C, followed by addition of 10 µl of concentrated sulfuric acid. This acid hydrolysis causes colorless flavan-4-ols to appear purple because of the formation of flavylium ions (Styles & Ceska, 1989). Total flavan-4-ols concentration was then determined by spectrophotometer using absorbance at 550 nm (λ_{max}) (Grotewold et al., 1998).

Analysis of excision footprints

The *Y1-cs* allele used in this study carries the *Cs1* transposon within the intron 2 of the *y1* gene. Primer positions within the intron 2 (forward primer, F) and exon 3 (reverse primer, R) used for the PCR amplification of *Cs1* excision footprints are shown in Figure 1b. Primer sequences are: forward F, 5′-TTGACACTGCGGACGCTGAG-3′, and reverse R, 5′-GAGTTCCAGTAGTTCTTGATC-3′. Standard PCR reaction conditions were followed (Sambrook and Russel, 2001) with the modified annealing temperature of 59°C for 2 min, followed by polymerization at 72°C for 2 min. PCR products were sub-cloned in pGEM-T-Easy vector (Promega, Madison, WI) and sequenced from both ends using vector specific primers. All DNA sequencings were done at the Pennsylvania State University's Nucleic Acid Facility using the method of dye primer cycle sequencing and reactions were run on a 3100 capillary machine (Applied Biosystems, Foster City, CA).

Results

Somatic excision of Cs1 *from* Y1-cs *is highly variable*

Genomic DNA from 100 individual seedlings from a line carrying the *Y1-cs-3* allele was analyzed. A representative gel blot carrying *Bam*HI digests was hybridized with the fragment F3 of the *y1* gene and results are presented in Figure 2a. The F3 probe fragment detects a restriction fragment length polymorphism (RFLP) within the y1 sequence and distinguishes between *Y1-rr* and *Y1-cs* alleles. For example, restriction enzyme digestion at the two *Bam*HI sites within the functional *y1* gene produces a 4.9 kbp wild type (*Y1-rr*) band in the absence of *Cs1* insertion (see Figure 1(b)). However, presence of the *Cs1* provides an additional *Bam*HI site which is present at the 5′ end of *Cs1* resulting in an approximately 8.0 kbp band. As a control for the 8.0 kbp band, we have used an unstable allele of the *Cs1* that is associated with the *Y1-cs-4* allele in which the *Cs1* rarely excises and this leads to the observance of very few revertant sectors in seed pericarp (Figure 2, lane 1). Genomic DNA from a germinal revertant stock RR-3 (*Y1-rr-3*) was used as a marker for the 4.9 kbp band (lane 9). Results indicate that, in the seedling, there is a high and variable rate of somatic excisions of *Cs1* from *Y1-cs* as measured by the presence/absence of the expected 4.9 and 8.0 kbp bands corresponding to the two allelic states of *Y1-rr* and *Y1-cs*, respectively.

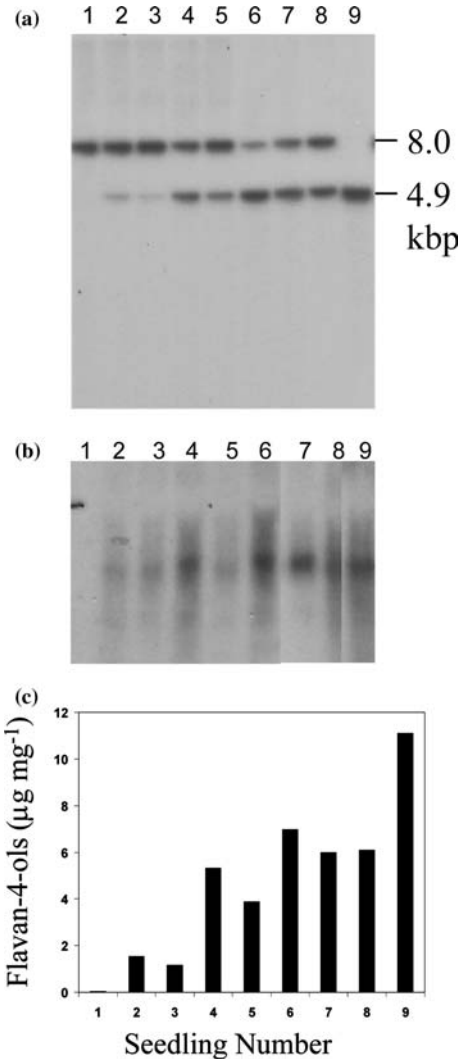

Figure 2. Somatic excision events of *Cs1* and functional characterization of *y1* locus in sorghum seedlings. (a) Representative Southern blot demonstrating somatic excision of *Cs1* element from the *Y1-cs* allele. Fragment F3 was used as a probe on a gel blot prepared from individual seedling leaf DNA digested with *Bam*HI. An approximately 8.0 kbp band represents a partial *y1* sequence carrying a portion of the *Cs1* element while the 4.9 kbp band corresponds to the revertant functional *Y1* allele (see Figure 1b for distribution of enzyme sites). Lane1, *Y1-cs-4* Lanes 2–8, *Y1-cs-3*; Lane 9, *Y1-rr-3*. (b) RNA was isolated from second portion of the leaf tissue of corresponding seedlings that were used to determine allelic states by RFLP. An y1 cDNA fragment was used as a probe which detects an approximately 1.9 kbp transcript. (c) Flavan-4-ols concentrations represent flavylium ions measured at A550 from acid-methanolic extract of third tissue portion of the same tissue used for DNA and RNA studies.

To confirm the excision of *Cs1* from *Y1-cs*, we measured expression of *y1* at RNA level. Hybridization signals with y1 cDNA as a probe showed varying levels of accumulation of y1 transcript in different seedlings (Figure 2a). As previously shown (Chopra et al., 1999), although Y1 is required for the biosynthesis of phlobaphenes in seed pericarp and mature leaves, the function of Y1 in seedling leaves is not known. Considering the fact that because of chlorophyll, phlobaphene pigments were not visible in the green leaves of sorghum (Zanta et al., 1994), we measured concentrations of flavan-4-ols (luteoferol and apiferol). Flavan-4-ols have previously been shown to be precursors of phlobaphenes in maize pericarp (Styles & Ceska, 1989; Grotewold et al., 1998). Results presented in Figure 2c confirm that the amounts of flavan-4-ols correlate with the allelic states observed by RFLP and RNA expression. These results thus corroborate the idea that in seedling leaves *Cs-1* has a variable somatic excision rate.

Identification of transposed copies of Cs1 *in red revertant plants*

Germinal excisions of the *Cs1* were followed by the identification of red revertant heads followed by confirmation through DNA gel blot analysis. As expected, most of these revertants were heterozygous (*Y1-rr*/*Y1-cs*) and their progenies showed a 3:1 segregation for red versus variegated pericarp phenotypes. Gel blots carrying restriction enzyme digests of *Y1-cs* and the revertant *Y1-rr* plants were used to determine restriction fragment length polymorphisms. A representative blot carrying DNA from *Y1-cs* and heterozygote revertant plants were hybridized with *Cs1* probes obtained either from the 5′ end (fragment F28) or the 3′ end (fragment F36) and results are presented in Figure 3(a). These probe fragments detect 6 to 7 copies of the probable full length *Cs1* element. New bands detected in the digests of the red revertant genomic DNA represent new copies of the *Cs1* insertion elsewhere in the genome. Using this criterion, we screened 340 plants obtained from a recombinant population (see materials and methods) and results obtained from a representative sub-set are shown in Figure 3b. These results show identification of red revertant (R) plants that show co-segregation of transposed *Cs1* (*Cs*1^{tr}) as well as the original copy of the *Cs1* element present in the *Y1-cs*.

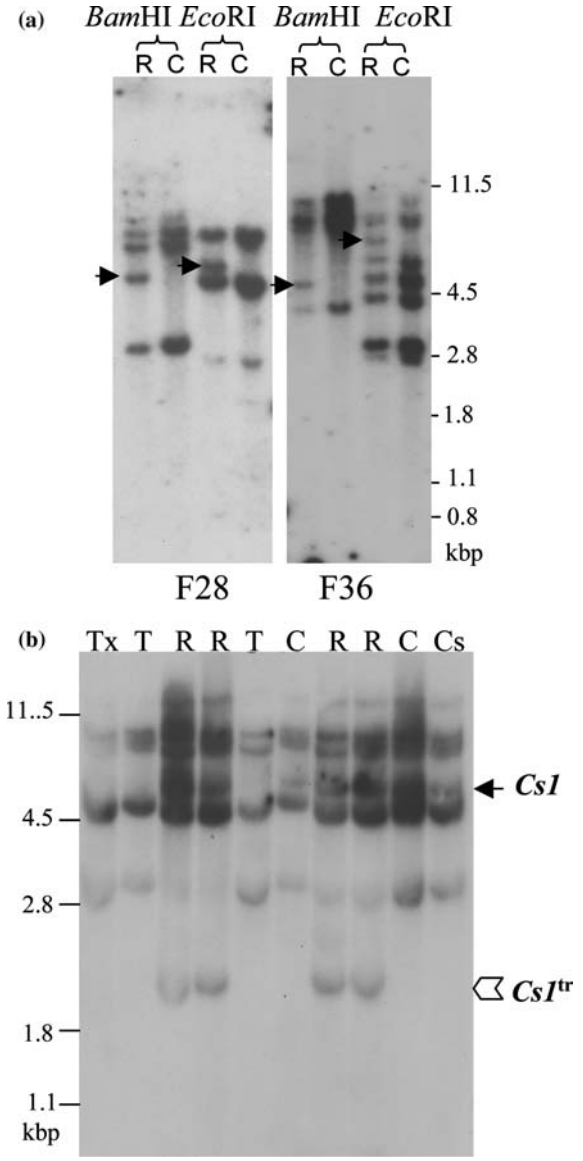

Figure 3. Germinal excision of *Cs1*. (a) Molecular characterization of siblings *Y1-cs-3* (C) and revertant *Y1-rr* (R) plants showing restriction patterns with *Cs1*- 5 (F28) and 3 (F36) fragments as probes. New *Cs1* copies obtained because of germinal excision and re-insertion elsewhere are shown by arrows in the revertant DNA digests. Molecular weight marker in kbp is shown. (b) A representative DNA gel blot showing excision and re-insertion of *Cs1* element from progeny of a red revertant. Restriction digests using *Eco*RI endonuclease are shown. Lanes Tx and Cs represent the parental lines Tx2737 and Cs8110419, respectively. Segregating alleles are: T, Tx2737; R, Red revertants (heterozygotes shown); and C, *Y1-cs* allele from CS8110419 genotype. An arrow indicates the position of the original *Cs1* element in the *Y1-cs* allele, while an open arrow head represents the position of the transposed $Cs1^{tr}$. Molecular weights are shown in kbp.

Cs1-excisions generate consensus 2 bp footprints

Footprints were examined from 15 seedling leaf and 12 immature pericarp DNAs of *Y1-cs* for somatic excisions and 18 *Y1-rr* seedling leaf DNAs for germinal excisions. Sequence characterization (Figure 4) shows that majority of the footprints had a two base pair sequence 'T/T' created during the excision of the *Cs1*. Three of the footprints (TCT/TCT) identified in the somatic excision events showed precise excision and this type of footprint was not recovered in the germinal excision events tested so far.

Mutant phenotypes and Cs1 *co-segregation analysis*

Approximately 8000 total plants were screened giving rise to 800 independent red revertants out of which 17 unique mutant phenotypes were identified. Mutants identified in this work have been given a description following the maize nomenclature (Neuffer, Coe & Wessler, 1997). The use of the same descriptive name in this study as used in maize however may not imply that the mutation found in sorghum is in the same gene as identified in maize. Mutant description is presented in Table 1, and a few selected phenotypes are shown in Figure 5. All

Allele	Event		Footprint Sequence		Frequency
Y1-cs	mutable	AGCAAG	**TCT** -*Cs1*-**TCT**	CCTCGA	
$Y1\text{-}rr^{wt}$	wt	5'-AGCAAG	**TCT**	CCTCGA-3'	
Y1-cs	Somatic excisions				
	Leaf	AGCAAG	**TT**	CCTCGA	12
		AGCAAG	**TCTTT**	CCTCGA	2
		AGCAAG	**TCTTCT**	CCTCGA	1
	Pericarp	AGCAAG	**TT**	CCTCGA	8
		AGCAAG	**TCTTT**	CCTCGA	2
		AGCAAG	**TCTTCT**	CCTCGA	2
$Y1\text{-}rr^{rev}$	Germinal excisions				
		AGCAAG	**TT**	CCTCGA	14
		AGCAAG	**TCTTT**	CCTCGA	4

Figure 4. Footprint analysis of somatic and germinal excisions of *Cs1*. Wild type *Y1-rr* allele sequence represents the *Cs1* target site (TCT). The mutable allele *Y1-cs* sequence is shown carrying the *Cs1* element flanked by the 5′ and 3′ copies of the duplicated target site sequence and is shown as 5′..TCT-*Cs1*-TCT...3′. Footprints left behind by somatic and germinal excisions of *Cs1* are shown with their frequency of occurrence.

of the progenies segregating for mutant phenotypes were grown for 2–3 generations to get inheritance data and chi-square analysis established recessive 3:1 (normal:mutant) Mendelian ratios for majority of the mutation (see Table 2).

We further used RFLP to perform co-segregation analysis between the mutant phenotypes and to determine any genetic linkage with the presence of *Cs1* hybridizing bands at new genomic locations. Mutants analyzed for the co-segregation analysis included: *old gold, pale green, third leaf yellow, zebra crossbands* and *lower leaf senescence*. In most cases segregating progeny rows were used to identify wild type homozygotes, heterozygotes and mutant homozygous plants. If a test cross progeny was available during genetic analysis, then normal and mutant homozygotes were used for molecular analysis. The third leaf from a 4 leaf stage plant was harvested for DNA extraction followed by preparation of DNA gel blots. Co-segregation analysis was performed using restriction enzymes near the 5′ and 3′ ends of the *Cs1* sequence and these included *Eco*RI, *Bam*HI, *Hin*dIII, *Sca*I, *Pst*I and *Kpn*I. Co-segregating bands of *Cs1* were identified for old gold and third leaf yellow. Third leaf yellow mutation segregated as a 3:1 recessive trait (see Table 2). Figure 6a shows a representative DNA gel blot hybridization pattern obtained from the third leaf yellow mutant. Similarly, chlorophyll-deficient old gold mutant also showed a 3:1 phenotypic ratio and a representative DNA gel blot is shown in Figure 6b. Both of the blots reveal bands highly homologous to the probes derived from *Cs1*. A 3.0 kbp *Eco*RI band co-segregates with the third leaf yellow mutant phenotype (panel a) and a 4.0 kbp *Sca*I band is present in the DNA of the chlorophyll deficient phenotypes in the old gold mutation (Figure 6b).

Discussion

The sorghum *y1* gene is a homologue of the maize *p1* and controls the biosynthesis of the red phlobaphene pigments in pericarp (Chopra et al., 1999; Zhang, Chopra & Peterson, 2000). Due to the insertion of the *Cs1*, the *y1* gene function is disrupted and the seed pericarp becomes colorless (Chopra et al., 1999). The red-white sectors or the

Table 1. Description and frequency of mutants screened from red revertants

Mutant	Phenotype	Viability	Freq.
Albino	White seedlings	Lethal	10
Bloomless	Absence of epicuticular wax; Figure 5c	Good	1
Brittle	Longitudinally corrugated leaves	Good	2
Brown midrib	Brown pigment in vascular bundles of leaves	Good	2
Dwarf	Short, compact plant with wrinkled leaves	Fair	2
Iojap striping	Variable white stripes/sectors on leaves	Poor	2
Lesion mimic	Small necrotic lesions on green leaves	Good	1
Male Sterile	Sterile pollen	Sterile	1
Old gold	Bright yellow stripes on leaves; Figure 5b	Good	8
Pale green	Pale green seedlings	Poor	3
Lower leaf senescence	Lower leaves show symptoms; Figure 5g	Poor	3
Rusty	Leaves with rusty lesions	Fair	1
Striate leaves	Leaves with many fine yellow stripes	Good	1
Third leaf light yellow	Third leaf of seedling light yellow. Bottom two leaves normal green; Figure 5f	Poor to fair	2
Yellow green	Leaves show yellow tissues between veins; Figure 5e	Poor	4
Wilty	Curled leaves with burned tips; Figure 5d	Good	2
Zebra crossbands	Light green cross bands on leaves; Figure 5a	Good	3

Viability indicates general health and vigor of the mutant compared with normal plant, and is categorized as good (as vigorous as a normal plant), fair (less vigorous than normal plants), poor (very weak plants) and lethal (no adult plants are produced).

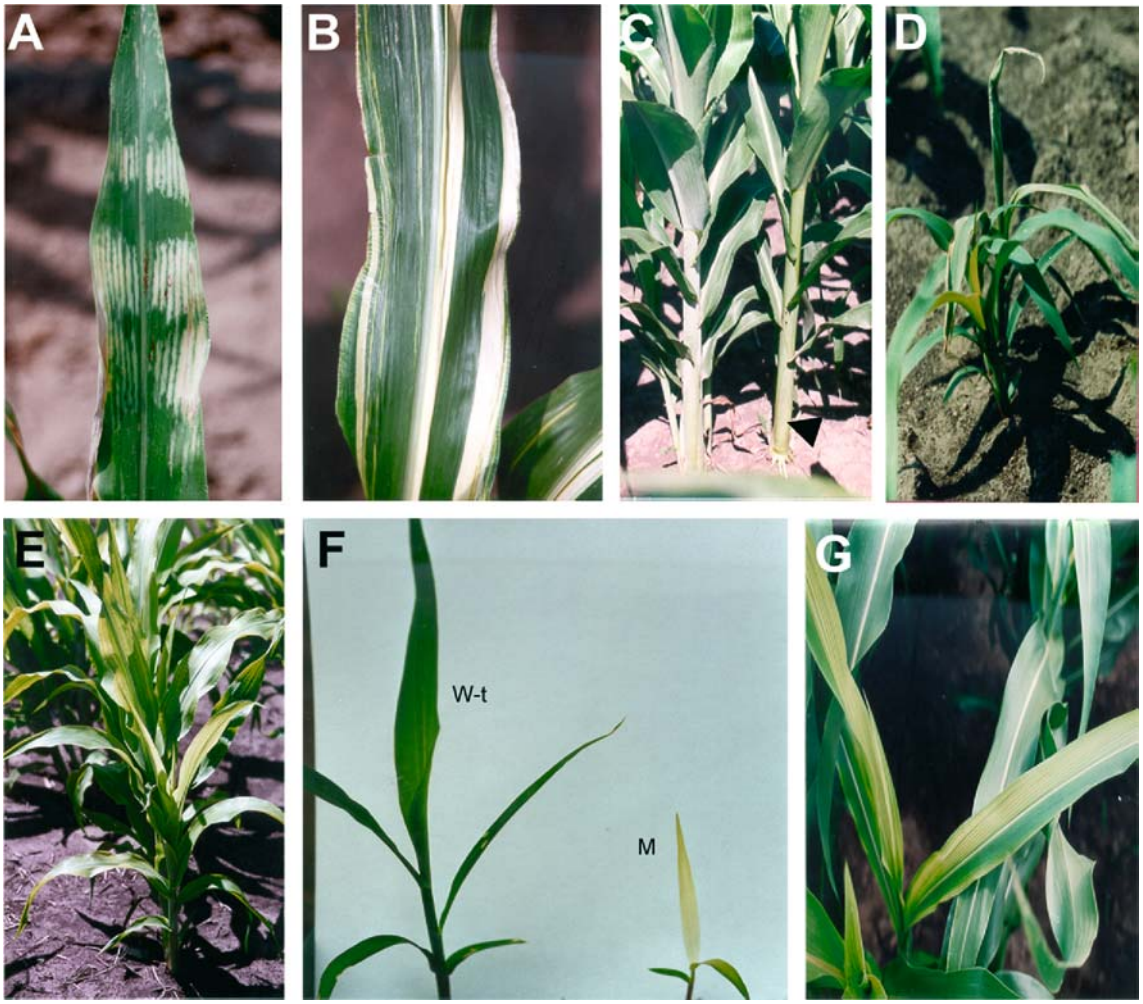

Figure 5. Phenotypes of selected mutants. (A) 'Zebra crossbands' showing chlorophyll less horizontal stripes; (B) 'Old gold' mutation with golden yellow stripes as oppose to 'iojap' mutation which has white stripes and large colorless leaf sectors (see Table 1); (C) 'Bloomless' mutant do not synthesize epicuticular wax (shown with an arrow) as compared to the wild type sibling plant (left); (D) 'Wilty' mutation has curled leaves and burned tips of top leaves; (E) 'Yellow green' mutation shows yellow tissue between veins; (F) 'Third leaf light yellow' mutation appears on seedling third leaf, while the two bottom leaves are normal green; and (G) 'Lower leaf senescence' mutation appears on the lower leaves only.

Table 2. Segregation analysis of selected mutations identified in the cross (CS8110419 × Tx2737)

Mutant phenotype	Normal plants	Mutant plants	χ^2 value
Brown midrib	158	31	7.45*
Bloomless	173	115	>10*
Old gold	198	55	1.43
Wilty seedling	685	154	>10*
Third leaf yellow	71	12	4.91
Dwarf growth habit (dominant)	37	72	4.65

* Significant deviation at 0.05 probability level for a 3:1 segregation.

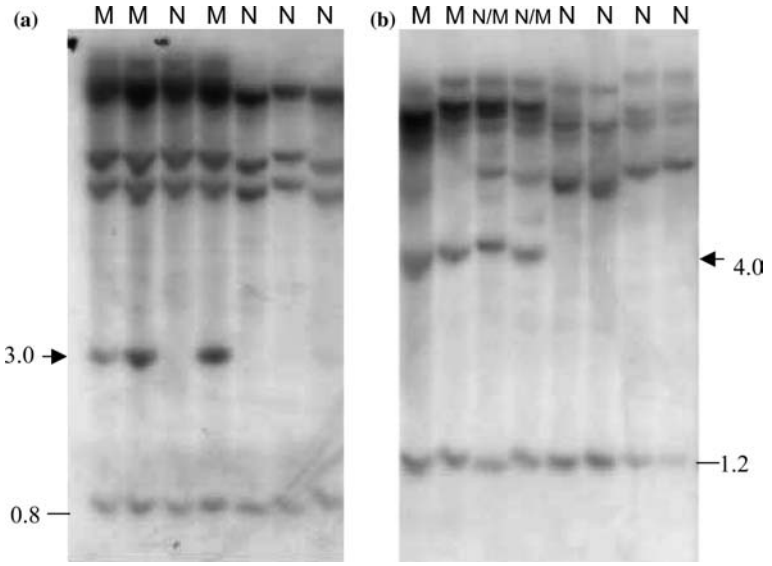

Figure 6. Co-segregation analysis of Third leaf yellow (a) and Old gold (b) mutants with *Cs1* transposon. Shown are representative Southern blots prepared from siblings showing mutant (M) and wild type (N) phenotypes which were hybridized with the 5′ end probe F28 (a) and 3′ end probe F36 (b). N/M plants represent heterozygous plants. Polymorphic and co-segregating bands of *Cs1* are indicated by an arrow with approximate molecular weight in kbp.

variegated pericarp phenotype results from the somatic excisions of the *Cs1* element from *y1*, while germinal excisions of the *Cs1* generate heritably functional alleles. In this study, we have exploited the variegated pigmentation phenotype as a marker and analyzed somatic and germinal excision events of the *Cs1* transposon. We have followed the traditional approach of monitoring the phenotype of the donor *Cs1* site i.e. *y1* conditioned phenotypes; similar approach has been used in several tagging experiments in which *Ac* from the *P1-vv* allele of the maize *p1* gene was used to perform mutagenesis (Chen, Greemblatt & Dellaporta, 1987; Moreno et al., 1992; Dellaporta & Moreno, 1996). Germinal excision of the *Cs1* from the *Y1-cs-3* allele occurs at a very high frequency reaching as high as 28% (Hu, Kofoid & Liang, 1991; Zanta et al., 1994). In the current study, identification of 800 red revertants (out of approximately 8000 plants) provides a fair estimation of 10% excision frequency. We found that the frequency of obtaining red revertants was higher in early segregating generations as compared to later generations obtained by selfing. Since, the activity of maize *En*/*Spm* elements has been shown to be epigenetically regulated (reviewed in Fedoroff, Sclappi & Raina, 1995), it is possible that certain epigenetic mechanisms are also playing a role in the stability of the *Cs1* and its activity.

The candystripe line CS811409 used in this study also has a very high frequency of somatic excision as measured by the presence of red seeds in an otherwise variegated panicle (Zanta et al., 1994). Somatic excisions as measured from the visual intensity of the functional *Y1-rr* DNA band ranged from 2 to 40%. RNA expression and biochemical analysis of the phlobaphenes precursors confirms the somatic excisions, but these data does not provide strong evidence of the number of early versus late excision events in a given leaf tissue. However, in the mature tissues showing revertant pigmentation phenotypes, the size and the shape of the stripe/sector does depend upon the developmental timing of transposition of *Cs1* . On average, a higher number of somatic excision events were recorded in pericarp (200 sectors/100 seeds) than in mature leaf (65/100) and these results support previous observations of developmentally late transpositions of *Cs1* (Chopra et al., 1999, 2002). As shown for the maize *Mu* elements, developmentally late transpositions have increased chance of passing through meiosis giving rise to higher germinal excision events (Walbot & Rudenko, 2002).

In addition to the Southern blot analysis of excision events, sequence characterization of the empty sites showed that excision of *Cs1* leaves a footprint. Since, *Cs1* is inserted in the 2nd intron of the *y1* gene, excision activity generating small footprints or small sequence alterations does not interfere with the expression of revertant functional alleles. The majority of the footprints recovered here show a di-nucleotide sequence (tt) both in the case of somatic and germinal excisions of *Cs1*. Other footprints observed are typical of CACTA elements and have been observed in previous studies (Bonas, Summer & Saedler, 1984; Schwarz-Sommer et al., 1985; Inagaki et al., 1994). Only a few cases of precise excision of *Cs1* were observed in which the footprint carried the target site duplication that flanked the empty site (TCTTCT). Interestingly such an excision event was not recovered from our germinal revertants and this type of somatic footprint has also been reported previously in the study of maize *wx-8::spm-I8* allele (Schwarz-Sommer et al., 1985). Moreover, during the course of this study, we also recovered 9 loss-of-function alleles at the *y1* locus and designated them as *y1-ww* (white pericarp and white glume; not shown). A detailed characterization of these *y1-ww* alleles will be presented elsewhere. In context of the present study, it is interesting to mention that three of the characterized *y1-ww* alleles show large deletions of the *Cs1* and *y1* sequence (V. Sangar and S. Chopra, unpublished). These stable nulls will be useful for characterization of rare events of excision behavior of *Cs1* and may provide insight into additional mechanisms of transposition mediated rearrangements or deletions (Schwarz-Sommer et al., 1985; Martin & Lister, 1989).

Our results show that the *Cs1* reinserts elsewhere in the genome as detected by the presence of new bands of *Cs1* in red revertants and all red revertants did not show visible mutant phenotypes. It is also possible that the actual rate of mutation was much higher than the one observed because many mutant phenotypes may not be easily identified under field conditions. It is expected that only in some events the transposed copy of the element would be jumping into a genic region and causing a mutation, as previously demonstrated in other transposable element systems (Izawa et al., 1997; Cowpertwaite et al., 2002). In addition, we observed variable frequency (up to 20%) for the absence of the new DNA bands corresponding to transposed *Cs1* in red revertant DNAs. In fact, in one of the classical genetic studies in maize, frequent loss of *En* transposon following excision during chromosomal replication has been observed (Dash & Peterson, 1994). Absence of transposed *Cs1* specific bands may be the result of rapid degradation since we did not detect involvement of any epigenetic influence (R. Sekhon and S. Chopra, unpublished).

Sorghum mutants recovered from our phenotypic screens were easily identifiable because of their striking phenotypes. The co-segregation results do not confirm but indicate a genetic linkage between the mutant phenotype and possible presence of the *Cs1* insertion in a gene leading to the mutant phenotype. Interestingly, however, several of the mutations did not show any co-segregation with *Cs1* probes. Since our co-segregation analysis is solely based on hybridizing *Cs1* probe sequences, we could not rule out the possibility that the actual mutation may be caused by a defective *Cs1* or a non-autonomous element belonging to the *Cs1* family that have conserved terminal repeats but highly diverged internal sequences. Further identification of tagged loci will provide clues to the exact nature of the mutations as well as the identification of *Cs1* homologues, if any. Overall, our results indicate that it may be possible to use *Cs1* as a tagging tool. Such forward transposon mutagenesis screens using *Ac, Mu*, and *En*/*Spm* elements have proven to be powerful genetic tools for the isolation of mutants and cloning of genes in plant species (Ramachandran & Sundaresan, 2001; Brutnell, 2002; Walbot & Rudenko, 2002). Since, *Candystripe1* homologous sequences are present in low copy number in different races of sorghum (Chopra et al., 1999) fewer outcrosses will be required to remove the mutations caused by other active copies, if any. This contrasts with the high copy number and highly mutagenic *Mu* elements in maize. Earlier genetic and molecular characterization of *Cs1* implicated that the autonomous property is linked with the *Y1-cs* allele (Zanta et al., 1994; Chopra et al., 1999) and our present study showed that the *Y1-cs* allele can be successfully used as a *Cs1* donor, reporter and an active site for new mutations.

There are several questions regarding the efficacy of this element as a tagging tool in sorghum

that need further research. First, although the activity of *Cs1* has been demonstrated in the *y1* locus, it is not yet known if *Cs1* is active in other loci. Characterization of some of our putative mutants showing sharp mutant versus revertant leaf sectors will reveal if *Cs1* is active in these somatic cells. The second question that needs thorough investigation is, if *Cs1* associated with the *Y1-cs* is the only active copy of the transposon. Third important unknown property is if *Cs1* transposing to linked or unlinked sites in the genome. In addition to seeking answers to these questions, identifying reporter alleles will be useful to monitor the activity of the autonomous *Cs1* element as has been done in other endogenous transposable element systems like *Ac*/*Ds* and *En*/*Spm*. Together with on-going extensive physical and linkage mapping and EST sequence database developments in sorghum, *Candystripe1* transposon may thus provide a means to develop a transposon tagging and functional genomics tool, which is currently lacking in sorghum.

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